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**Differential expression of gonadotropin and estrogen receptors and oocyte cytology during follicular maturation associated with egg viability in European eel (*Anguilla anguilla*)**

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## Abstract

In captivity, oogenesis and ovarian follicle maturation in European eel can be induced experimentally using hormonal therapy. The follicle's ability to respond effectively to the induction of maturation and ovulation, resulting in viable eggs, depends on the oocyte stage at the time of induction. We hypothesized that variation in the expression of key hormone receptors in the ovary and size of oocyte lipid droplets are associated with changes in oocyte stage. Thus, we induced ovarian follicle maturation using a priming dose of fish pituitary extract followed by an administration of  $17\alpha$ ,  $20\beta$ -dihydroxy-4-pregnen-3-one (DHP). Females were then strip-spawned, the eggs were fertilized *in vitro*, incubated and larval survival was recorded at 3 days post hatch (dph). The expression of gonadotropin receptors (*fshr*, *lhcr1* and *lhcr2*) and estrogen receptors (*esr1*, *esr2a*, *esr2b*, *gpera* and *gperb*) was quantified and the size of oocyte lipid droplets measured. Larval survival at 3 dph was used to differentiate high- and low-quality egg batches. Results showed significantly higher abundance of *lhcr1* and *esr2a* at priming for high-quality egg batches whereas *fshr* and *gperb* transcripts were significantly higher at DHP injection for low-quality egg batches. Therefore, high levels of *lhcr1* and *esr2a* may be important for attaining follicular maturational competence, while high *fshr* and *gperb* mRNA levels may indicate inadequate maturational competence. Furthermore, lipid droplet size at DHP and in ovulated eggs was significantly smaller in high-quality egg batches than in low-quality, which indicates that droplet size may be a useful marker of follicular maturational stage.

**Key words:** estrogen receptors, gonadotropin receptors, lipid droplet, follicular maturation, egg quality

## 1. Introduction

The oceanic, reproductive stages of European eel (*Anguilla anguilla*) remain undiscovered and sexual maturation neither occurs naturally in their continental habitats nor in captivity. This arrested development results from a strong dopaminergic inhibition (Dufour et al., 1988; Vidal et al., 2004) and a deficient pituitary gonadotropic function, i.e. both gonadotropin synthesis and release are low (Dufour et al., 1983). Experimentally, gonadal development can be induced using hormonal treatments based on fish pituitary extracts in females and human chorionic gonadotropin in males (Fontaine et al. 1964; Yamamoto and Yamauchi, 1974; Dufour et al., 1989; Ohta et al., 1996; Pedersen, 2003). In females, such treatment leads to oocyte growth until maturation. Induction of follicular maturation and ovulation generally requires an additional dose of pituitary extract and an injection of a maturation-inducing steroid (MIS) (Yamauchi, 1990; Ohta et al., 1996; Pedersen, 2003) such as 17 $\alpha$ , 20 $\beta$ -dihydroxy-4-pregnen-3-one (DHP) in the case of the eel. Recent advances in eel assisted reproductive technology have enabled the production of viable eggs and yolk-sac larvae (Butts et al., 2016; Sørensen et al., 2016). However, resulting egg quality is variable and low fertilization rate and poor larval survival are often observed. Variation in egg quality is partly related to the timing induction of ovarian follicle maturation and ovulation, as previous studies have shown that hormonal treatment given too early or too late in the reproductive cycle can be ineffective or inefficient (Palstra et al. 2005; Mylonas et al., 2010; Unuma et al., 2011). Because hormones need to bind to receptors to exert their biological function, differences in responsiveness to hormonal treatment could be due to differences in hormone receptor expression.

In teleost fishes, as in other vertebrates, the two pituitary gonadotropin hormones follicle-stimulating hormone (FSH) and luteinizing hormone (LH) play major roles in the regulation of oogenesis and

62 production of sex steroids (Pierce and Parsons, 1981). Gonadotropic function is mediated by specific  
63 membrane receptors, the FSH receptor (FSHR) and the LH receptor (LHCGR), which corresponds to  
64 the human LHCGR. These are mainly expressed in the somatic cells of the gonads (Rosenfeld et al.,  
65 2007), though they can also be expressed in germ cells (Chauvigné et al., 2014). Until recently, it was  
66 accepted that teleosts had a single FSHR and a single LHCGR encoded by *fshr* and *lhcr* genes,  
67 respectively. However, in addition to *fshr*, Maugars and Dufour (2015) identified and characterized two  
68 *lhcr* genes (*lhcr1* and *lhcr2*) in some fish species, including the European eel. European eel *lhcr1*  
69 corresponds to the LHCGR previously characterized in the Japanese eel (*Anguilla japonica*) (Kazeto et  
70 al., 2012). In comparison to the many studies on FSH and LH, knowledge about their receptors in  
71 teleost species is still limited.

72 Sex steroids, such as estrogens, are also well-known for their role in reproductive function. In female  
73 fish, estrogens (primarily estradiol-17 $\beta$ , E2) are involved in the regulation of oogenesis and  
74 vitellogenesis (Wallace, 1985) as well as in the feedback control of gonadotropin expression and  
75 release (Zohar et al., 2010). Estrogens can diffuse through the cell membrane and bind to nuclear  
76 estrogen receptors (ESRs). Two distinct subtypes of nuclear ESRs, *esr1* and *esr2*, have been cloned  
77 from several mammalian and non-mammalian vertebrates (Mosselman et al. 1996; Hawkins et al.,  
78 2000). In teleosts, including the European eel, two paralogs of *esr2* have been reported, *esr2a* (also  
79 named *er $\beta$ 2*) and *esr2b* (also named *er $\beta$ 1*) (Hawkins et al., 2000; Ma et al., 2000; Menuet et al., 2002;  
80 Nagler et al., 2007; Lafont et al., 2016). In addition, estrogens can also activate receptors on the cell  
81 surface, initiating rapid and often non-genomic biological responses (Watson and Gametchu, 1999;  
82 Falkenstein et al., 2000; Norman et al., 2004). The human G-protein coupled receptor 30 (GPER30 or  
83 GPER) was shown to have the binding characteristics of an E2 membrane receptor (Revankar et al.,

2005; Thomas et al., 2005). Subsequent studies in zebrafish, Atlantic croaker (*Micropogonias undulatus*) and common carp (*Cyprinus caprio*) have also shown that estrogens produced by follicle cells inhibit or delay spontaneous follicular maturation via the activation of membrane receptors (Pang et al., 2008; Pang and Thomas, 2009; Peyton and Thomas, 2011; Majumder et al., 2015). In the European eel and some other teleosts, two paralogous *gper* genes (*gpera* and *gperb*) have been recently identified (Lafont et al. 2016) but their roles have not yet been investigated.

During follicular maturation, cytological changes in the oocytes take place. This includes migration of the germinal vesicle towards the oocyte periphery followed by breakdown of the nuclear envelope, and meiotic resumption (Lubzens et al., 2017). In many teleosts, including the eel, another feature of oocyte cytoplasmic maturation is the coalescence of lipid droplets to form one or a few large oil globules (Kagawa, 2013). These cytological changes have been used as biomarkers for assessment of oocyte maturational status in relation to assisted reproduction of eel (Palstra et al., 2005; Unuma et al., 2011).

The main objective of this study was to assess the expression of gonadotropin receptors (*fshr*, *lhcr1* and *lhcr2*) and estrogen receptors (*esr1*, *esr2a*, *esr2b*, *gpera* and *gperb*) during induced maturation and ovulation and investigate their relation with subsequent egg quality, ultimately estimated as fertilization rate, hatching success and early larval survival. Finally, concomitant changes in oocyte lipid droplet size were evaluated as potential cytological biomarker of follicular maturational status.

## 2 Materials and Methods

### 2.1 Ethics statement

All fish were handled in accordance with the European Union regulations concerning the protection of experimental animals (Dir 86/609/EEC). Eel experimental breeding protocols were approved by the

105 Animal Experiments Inspectorate (AEI), Danish Ministry of Food, Agriculture and Fisheries (permit  
106 number: 2010/561-1783). All efforts were made to minimize animal handling and stress.

## 107 2.2 *Experimental animals and rearing conditions*

108 Female silver eels (n=10; mean length and weight  $\pm$  SD were  $72 \pm 11$  cm and  $781 \pm 393$  g,  
109 respectively) were caught from a freshwater lake (Vandet Sø) in northern Jutland (Denmark) and  
110 transported to a research facility of the Technical University of Denmark located at Lyksvad Fish Farm  
111 (Vamdrup, Denmark). Eels were randomly distributed into duplicate 300 L tanks equipped with a  
112 recirculation system and gradually acclimated to artificial saltwater over a period of two weeks, i.e.  
113 freshwater adjusted to 36 ppt salinity using Tropic Marin Sea Salt (Dr. Biener GmbH, Wartenberg,  
114 Germany). Thirty male eels (body weight  $106 \pm 13$  g; body length  $38 \pm 2$  cm) reared on DAN-EX  
115 2848 (BioMar A/S, Brande, Denmark) were obtained at a commercial eel farm (Stensgård Eel Farm  
116 A/S, Randbøl, Denmark), transported to Lyksvad Fish Farm and kept in separate tanks under the same  
117 conditions as the female eels. At the onset of hormonal treatment, the eels were anaesthetized  
118 individually in an aqueous solution of benzocaine (ethyl p-aminobenzoate, 20 mg/L, Sigma-Aldrich,  
119 Germany), tagged with a passive integrated transponder (pit-tag) in the abdominal muscle, and body  
120 weight and length were measured. Throughout the experiment, all fish were maintained at ~36 ppt  
121 salinity, ~20 °C, and a natural local daily photoperiod. No feed was provided during experiments since  
122 eels in the migratory, silvering stage cease feeding (Lokman et al., 2003).

## 123 2.3 *Induction of gametogenesis and sampling*

124 Females received weekly intramuscular injection of salmon pituitary extract (SPE) at a constant dosage  
125 of 18.75 mg/kg initial body weight to induce and sustain follicular development and vitellogenesis  
126 (Kagawa et al., 2005; Tomkiewicz et al., 2012). The first injection was given concurrent with pit-

127 tagging and regular treatment lasted 16-20 weeks depending on the responsiveness of the females.  
128 Pituitary extract was prepared using freeze-dried salmon pituitaries (Argent Chemical Laboratories,  
129 Washington, USA) that were grinded, diluted in NaCl 0.9 g/L and centrifuged according to Ohta et al.  
130 (1996, 1997). Supernatants were stored at -20 °C until use. Females were weighed at the weekly  
131 injections to follow changes in body weight.

132 Individual treatment for follicular maturation and ovulation was initiated at first signs of the onset of  
133 oocyte hydration, i.e. a body weight increase of 10 – 15 % compared to the initial weight and a soft  
134 abdomen (Pedersen, 2003, 2004). To assess oocyte developmental stage, each female was  
135 anaesthetized in an aqueous solution of benzocaine, and an ovarian biopsy (~0.2 ml) was obtained,  
136 using a sterile disposable injection needle (16G x 1 ½”). The biopsy was taken at a standard location on  
137 the left side of the body ~5 - 10 cm anterior to the genital pore, relative to female size, and the female  
138 thereafter transferred to a separate tank under the same conditions for individual care. The biopsy was  
139 inspected under the microscope and oocyte development graded on a scale from 1-7 according to  
140 Palstra et al. (2005). Progression of oocyte maturation varied in time and homogeneity, so each female  
141 was followed until the most developed oocytes exhibited characteristics close to stage 4, i.e., fully  
142 transparent oocyte with nucleus at periphery (Fig. 1). At this stage, an additional SPE injection as  
143 primer was given to females to sustain and boost follicular development (Pedersen, 2004; Kagawa et  
144 al., 2005). To complete follicular maturation and induce ovulation, the eel maturation inducing steroid,  
145 17 $\alpha$ , 20 $\beta$ -dihydroxy-4-pregnen-3-one (DHP crystalline, Sigma-Aldrich Chemie, Steinheim, Germany)  
146 was given ~24 h later at a dose of 2 mg/kg present body weight (Ohta et al., 1996). Prior to DHP  
147 injection, a new biopsy (~0.2 ml) was obtained to evaluate the progression of oocyte development.  
148 DHP was injected into the ovarian tissue under anaesthesia (Palstra et al. 2005). Female eels ovulated



149 12 to 15 hours after DHP injection and at that time the eggs were stripped by applying gentle pressure  
150 along the abdomen of the fish. The volume of ovulated eggs collected was recorded.

151 Induction of spermatogenesis in the male eels started 4 weeks after the induction of oogenesis in  
152 females, as males only need 7-9 weeks of treatment to reach spermiation (Tomkiewicz et al. 2011).  
153 Each week, males received an intramuscular injection of human chorionic gonadotropin (hCG, Sigma  
154 Aldrich Denmark, A/S) at dose of 1.5 IU hCG per kg initial body weight (Butts et al., 2014). An  
155 additional hCG injection was given 12 h prior to milt collection in order to enhance sperm production.

#### 156 2.4 *Sampling for gene expression and cytological analysis*

157 Sampling for each female comprised: 1) the ~0.2 ml ovarian biopsy obtained ~1h before the SPE  
158 priming injection (SPE); 2) the ~0.2 ml biopsy obtained prior to DHP injection (DHP) and 3) a sample  
159 of unfertilized eggs collected immediately after stripping (EGG). Each sample included digital images  
160 of oocytes/eggs to measure lipid droplet diameter and a preserved sample (~0.1 ml) for gene expression  
161 analysis. Prior to photography, the sampled oocytes/eggs were cleared in Serra's liquid  
162 (ethanol:formalin:acetic at 6:4:1, diluted 20X in phosphate buffered saline, PBS, see Stoeckel (2000))  
163 for staining of the germinal vesicle. The pictures were taken at 20X magnification using a digital  
164 camera (Digital Sight DS-Fi1, Nikon Corporation, Japan) connected to an optical microscope (Eclipse  
165 55i, Nikon Corporation, Japan) for measurement of lipid droplet diameter inside the oocytes. Samples  
166 taken for analysis of gene expression were preserved in RNA-later (Ambion Inc., Huntingdon, UK),  
167 refrigerated at 5 °C for 24 h and then frozen at -20 °C until RNA extraction.

168 2.5 *Fertilization rate, hatching success and larval survival*

169 In order to evaluate the egg viability, eggs were fertilized *in vitro*, and fertilization rate, hatching  
170 success, and larval survival was determined. For each female, milt from 4-5 males was collected,  
171 leaving three week intervals between individual male stripping. The spermatocrit was estimated  
172 (pooled milt mixture according to Sørensen et al. (2013) and used to dilute milt in an immobilizing  
173 diluent (Asturiano et al., 2004; Peñaranda et al., 2010). The diluted milt at a standardized concentration  
174 was used for fertilization within 4 hours after collection (Butts et al., 2014).

175 Immediately after stripping, eggs were mixed with the milt solution and added natural seawater for  
176 sperm activation (Butts et al. 2014). After 5 min gamete contact time, a sub-sample of eggs was  
177 transferred to a 100 ml graduated cylinder glass to estimate percent floating eggs. The remaining eggs  
178 were transferred to 10 L containers for separation of floating and sinking eggs. The eggs were kept at  
179 20 °C and the fertilization rate and floating percent was accessed 3-5 hours post fertilization (HPF). To  
180 estimate fertilization success, a sample of 100-150 floating eggs was photographed using an optical  
181 microscope (Eclipse 55i, Nikon Corporation, Japan) at 20X magnification and a digital camera (Digital  
182 Sight DS-Fi1, Nikon Corporation, Japan). Fertilized eggs were identified by the presence of blastomere  
183 cleavage (minimum 4 cell stage), while those that had not reached the 4-cell stage were considered  
184 unfertilized.

185 For estimation of hatching success, eggs (~200 eggs in triplicate, for each female) were collected from  
186 the floating layer after 3-5 HPF and incubated at 20 °C in flasks (Nunc® 75 cm<sup>2</sup> flasks, non-treated  
187 with ventilated caps, Thermo Scientific) (Sørensen et al. 2014). Each flask contained 250 ml of  
188 seawater (36 ppt) ampicillin (50 mg/L) and rifampicin (50 mg/L). Flasks stayed undisturbed inside a  
189 dark and closed incubator at 20 °C until the number of hatched larvae was counted at 55 HPF. To

190 estimate larval survival, ~300 g of eggs from the floating layer were incubated in a 60 L incubator with  
191 natural seawater at ~20 °C. After hatch, when available ~3000 larvae in triplicate were stocked in 40 L  
192 tanks of an aquaculture recirculation system containing natural seawater adjusted to 36 ppt salinity  
193 using Tropic Marin Sea Salt and reared at 20 °C. Survival was measured as the percentage of still  
194 living larvae at 3 days post hatch (dph).

195 For statistical analysis, egg batches were categorized into two groups: i) high-quality and ii) low-  
196 quality based on larval survival. Hereby, survival beyond the first 3 dph (high mortality period) was  
197 used to differentiate the groups (Table 2).

## 198 2.6 *Gene expression analysis*

### 199 RNA extraction and cDNA synthesis:

200 After Proteinase K treatment, total RNA was purified from ovarian biopsies and ovulated eggs by  
201 homogenization of 30 mg of tissue in 700 µl TRIzol reagent (Invitrogen Life Technologies, Carlsbad,  
202 CA, USA). TRIzol/chloroform separation was performed to remove DNA and proteins. The aqueous-  
203 phase was transferred to a new tube and 500 µl of isopropanol were added for RNA precipitation.  
204 Genomic DNA contamination was removed by treating the total RNA with the TURBO DNA-free kit  
205 (Ambion). Total RNA quality and concentration was measured spectrophotometrically using  
206 NanoDrop (Saveen Werner AB, Limhamn, Sweden).

207 RNA was then reverse transcribed into cDNA using Hight Capacity RNA-to-cDNA kit (AB) according  
208 to manufacturer's protocol. In summary, reaction volumes of 20 µl contained 450 ng total RNA, 1X RT  
209 buffer, 1X enzyme mix. The following cycling parameters were used: 37 °C for 60 min, 95 °C for 5  
210 min and hold at 4 °C, using a 2720 Thermal cycler, AB.

211 Quantitative real-time PCR (qPCR):

212 The following gene specific primers were previously designed based on the nucleotide sequence of the  
213 European eel: gonadotropin receptors, *fshr*, *lhgr1* and *lhgr2* (Maugars and Dufour, 2015); estrogen  
214 receptors, *esr1*, *esr2a*, *esr2b*, *gpera* and *gperb* (Lafont et al., 2016). Specific primers for European eel  
215 18S ribosomal RNA gene (*18S*) were designed in this study (Table 1), and their specificity and  
216 efficiency was tested in qPCR.

217 The qPCRs were performed with a lightcycler (Roche, Ltd. Basel, Switzerland), using SYBR Green I.  
218 Each reaction was prepared with 4 µl of diluted cDNA template, 2 µl PCR grade water, 2 µl of SYBR  
219 Green master mix and 1 µl of each forward and reverse primers (0.5 pmol each at final concentration).

220 The following qPCR conditions were applied: polymerase activation step of 10 min at 95 °C, followed  
221 by 51 cycles of 10 sec of denaturizing at 95 °C, 5 sec of annealing at 60 °C, 6 sec of elongation at 72  
222 °C for *esr1*, *esr2a*, *esr2b*, *gpera* and *gperb* or 10 sec at 72 °C for *18S*, *lhgr1*, *lhgr2* and *fshr*. The  
223 programs ended with a melting curve analysis by slowly increasing the temperature (0.1 °C/sec) from  
224 65 °C to 95 °C, with a continuous registration of changes in fluorescent emission intensity. This last  
225 step aimed at ensuring the presence of only one amplified product. Each qPCR run contained a non-  
226 template control (cDNA was substituted by water) for each primer pairs to confirm that the qPCR mix  
227 was not contaminated. Serial dilutions of a pool of ovary biopsies and ovulated egg cDNAs were used  
228 as a standard curve for each gene. One dilution was included in each run as a calibrator. Normalization  
229 of the qPCR data was performed using *18S* as a reference gene since it was stable through all sampling  
230 points with a coefficient of variance at SPE, DHP and OV of 40%, 40% and 48%, respectively.

## 231 2.7 *Measurement of lipid droplet diameter*

232 Using the digital images of each sample (before SPE priming, before DHP injection and ovulated  
233 eggs), 10 oocytes were randomly selected among those at the most advanced stage of development.  
234 Here, ten of the largest lipid droplets were measured using the free software ImageJ (1.48d) and the  
235 maximum five values averaged (Unuma et al., 2011). For each lipid droplet, the diameter was  
236 calculated by the average of two diameter measurements. At the final stages of coalescence, only a few  
237 droplets became larger while the others became smaller (Fig. 1), in these stages, the diameter was  
238 based on the diameter of the largest droplets only (Unuma et al., 2011).

## 239 2.8 *Statistical analysis*

240 Statistical analysis was performed using R version 3.1.3 (R core team, 2015). Statistical differences in  
241 gene expression and lipid droplet diameter between quality groups (high- and low-quality) in relation to  
242 sampling time (before SPE, before DHP and after stripping) were evaluated using linear mixed-effects  
243 (LME) models. The female ID was included in the models as within-subjects variable to account for  
244 the repeated measurements taken on each female through the sampling points. Model assumptions of  
245 normality and equal variance were checked using Q-Q plots and by observation of the residuals versus  
246 fitted values plot. Square root and logarithmic transformations were applied to gene expression and  
247 droplet diameter data whenever data deviated from a normal distribution. Linear regression analysis  
248 was used to determine whether there were significant correlations between gene expression at each  
249 sampling time and fertilization rate and hatching success. The expression of each gene was screened for  
250 outliers using the Tukey's method. This method uses the interquartile range (IQR) to identify the  
251 outliers range above and below the 1.5X IQR. A significance level ( $p$ ) of 0.05 was applied in all tests.

### 3 Results

#### 3.1 Egg production, fertilization and hatching success

The females studied produced on  $327 \pm 152$  g eggs corresponding to 29-56 % of the initial body weight (Table 2). The percent floating eggs varied from 25 to 100 %, while the fertilization rate ranged between 15 to 99 % and the hatching rate between 0 to 80 %. Batch size was positively related to initial female body weight ( $R = 0.94$ ;  $p < 0.001$ ) and length ( $R = 0.95$ ;  $p < 0.001$ ). Fertilization rate was not correlated with female length ( $R = -0.03$ ;  $p = 0.930$ ) nor initial weight ( $R = 0.09$ ;  $p = 0.798$ ). Hatching success ranged from 19 to 80 % and 0 to 5 % in the high- and low-quality groups, respectively (Table 2). There was also no significant correlation between hatching success and female length ( $R = -0.03$ ;  $p = 0.927$ ) nor initial weight ( $R = 0.05$ ;  $p = 0.901$ ).

#### 3.2 Expression of gonadotropin receptors

All three gonadotropin receptors (*fshr*, *lhcgrr* and *lhcgrr2*) were detected in the ovarian biopsies with genes differentially expressed across sampling points from the induction of oocyte maturation, SPE and DHP, and EGG (Fig. 2). Overall, transcript levels of *fshr* did not vary during oocyte maturation, i.e. between the ovarian biopsy taken before SPE and the biopsy taken before DHP ( $p > 0.05$ ). However, *fshr* levels dropped down below the level of detection of qRT-PCR detection in the eggs (at least 534 times less expressed than in the ovarian biopsies). Within sampling points, the expression levels of *fshr* were similar between the high- and low-quality groups in the biopsies obtained prior to SPE priming ( $p > 0.05$ ; Fig. 2). However, before DHP, *fshr* transcript levels were significantly lower in the high-quality group than in the low-quality group ( $p < 0.001$ ). Present results also indicated a significant negative correlation between *fshr* before DHP injection and hatching success ( $R = -0.79$ ;  $p = 0.012$ ; Table 3).

273 Overall, *lhgr1* mRNA levels did not vary significantly ( $p > 0.05$ ) during maturation (from SPE to  
274 DHP injection). In contrast, transcript levels were around 150 times lower in ovulated eggs than in the  
275 ovarian biopsies ( $p < 0.001$ ). Moreover, in the ovarian biopsies collected before SPE priming, *lhgr1*  
276 expression was significantly higher in the high-quality group compared to the low-quality ( $p$   
277  $= 0.002$ ; Fig. 2). Present results also showed a significant positive correlation between *lhgr1* at SPE  
278 and hatching success ( $R = 0.68$ ,  $p = 0.032$ ; Table 3). There was no difference between the two groups  
279 in the ovary samples taken before DHP injection and in ovulated eggs ( $p > 0.05$ ).

280 Regardless of egg quality, *lhgr2* transcript levels were similar between the ovarian biopsies taken  
281 before SPE and DHP ( $p > 0.05$ ) but significantly less expressed in the ovulated eggs (at least 5 times  
282 less expressed than in the ovarian biopsies,  $p < 0.001$ ). Within sampling points, transcript levels of  
283 ovarian *lhgr2* at SPE priming and DHP injection did not differ significantly between the high and low  
284 quality groups ( $p > 0.05$ ; Fig. 2). However, *lhgr2* mRNA levels in unfertilized egg samples were  
285 significantly higher in the high-quality than in the low-quality group ( $p = 0.013$ ). There were no  
286 significant correlations between *lhgr2* transcript levels and hatching success or fertilization rate ( $p >$   
287  $0.05$ ; Table 3).

### 288 3.3 Expression of estrogen receptors

289 The nuclear receptor *esr1* was expressed in the ovarian samples obtained during oocyte maturation  
290 without significant variation between the ovarian samples before SPE priming and DHP ( $p > 0.05$ ; Fig.  
291 3). After stripping, transcript levels were below the level of detection of qRT-PCR in unfertilized eggs  
292 (at least 40 times less expressed than in the ovarian biopsies). Moreover, *esr1* expression was similar  
293 between the high- and low-quality groups within the SPE and DHP sampling points ( $p > 0.05$ ). There

294 were no significant correlations between *esr1* and hatching success or fertilization rate ( $p > 0.05$ ; Table  
295 3).

296 From the two nuclear *esr2* receptors, only *esr2a* was detected in the ovary during the induction of  
297 oocyte maturation. Irrespective of hatching success, transcript levels of *esr2a* did not vary during  
298 maturation treatment ( $p > 0.05$ ), but the gene was much less expressed in unfertilized eggs (at least 67  
299 times less expressed than in the ovarian biopsies,  $p < 0.001$ ). In the biopsies taken before SPE priming,  
300 transcript levels of *esr2a* were significantly higher in the high-quality group compared to the low-  
301 quality group ( $p = 0.020$ ; Fig. 3). In the ovarian biopsies taken before DHP and ovulated eggs, *esr2a*  
302 expression levels were similar between groups ( $p > 0.05$ ). We did not find any significant correlations  
303 between *esr2a* transcript levels and hatching success or fertilization rate ( $p > 0.05$ ; Table 3). Transcript  
304 levels of *esr2b* were below the qRT-PCR detection threshold in all samples and could not be measured.

305 The two estradiol membrane receptors, *gpera* and *gperb*, were detectable and presented a different  
306 expression pattern during induced oocyte maturation and after stripping (Fig. 3). Irrespective of egg  
307 quality group, *gpera* transcript levels did not vary during maturation treatment ( $p > 0.05$ ) but were  
308 significantly lower in the eggs ( $p < 0.001$ ). Expression of *gpera* did not differ significantly between  
309 high- and low-quality in neither the ovarian biopsies before SPE and DHP injection, nor in ovulated  
310 eggs ( $p > 0.05$ ; Fig. 3). There were no significant correlations between *gpera* transcript levels and  
311 hatching success or fertilization rate ( $p > 0.05$ ; Table 3). Overall, there was no significant difference in  
312 *gperb* mRNA expression levels during maturation ( $p > 0.05$ ) while transcript levels of *gperb* dropped  
313 to a level below the qRT-PCR detection threshold in ovulated eggs (at least 375 times less expressed  
314 than in the ovarian biopsies). The expression levels of *gperb* before SPE administration did not differ  
315 between the high-quality and low-quality group ( $p > 0.05$ ) while levels were significantly lower in the



316 high-quality before the DHP administration ( $p = 0.010$ ; Fig. 3). Present results did not show any  
317 significant correlations between *gperb* levels and hatching success or fertilization rate ( $P > 0.05$ ; Table  
318 3).

#### 319 3.4 Lipid-droplet related oocyte maturation status

320 Image analysis of the ovarian biopsies collected during the course of oocyte maturation induction  
321 revealed 2-3 cohorts of oocytes of different sizes and stages of development (Fig. 4). Oocytes at the  
322 most advanced stage of development were at the germinal vesicle migration stage, characterized by a  
323 transparent cytoplasm and peripheral germinal vesicle visible before SPE and DHP injection (Fig. 1a  
324 and b), similar to stage 4 and 5 according to the classification developed by Palstra et al. (2005),  
325 respectively. No germinal vesicle could be observed in ovulated eggs after clearing in Serra's liquid  
326 (Fig. 1c). As lipid droplets coalesced, their diameter increased significantly throughout oocyte  
327 maturation and stripped eggs ( $p < 0.001$ ). In the high-quality group, mean  $\pm$  SD lipid droplet diameter  
328 was  $89 \pm 23 \mu\text{m}$  at SPE priming,  $136 \pm 14 \mu\text{m}$  at DHP injection and  $156 \pm 15 \mu\text{m}$  at EGG. In the low-  
329 quality group, lipid droplet diameter was  $110 \pm 34 \mu\text{m}$  at SPE priming,  $194 \pm 72 \mu\text{m}$  at DHP injection  
330 and  $248 \pm 74 \mu\text{m}$  at EGG. Overall, mean lipid droplet diameter was significantly higher and with wide  
331 size variations in the low-quality than in the high-quality group ( $p = 0.028$ ; Fig. 5), and mean size  
332 increased significantly over time in both groups (from SPE to EGG,  $p < 0.001$ ). Lipid droplet size in  
333 the low-quality group also demonstrated an increasingly larger inter-female size variation over time,  
334 compared to the high-quality group, where there were less lipid droplet size variation at all times. We  
335 did not find a significant difference in droplet diameter between high- and low-quality at SPE priming  
336 ( $p > 0.05$ ) but at DHP and EGG, lipid droplet diameter was significantly higher in the low-quality  
337 group ( $p = 0.042$  and  $p = 0.005$ , respectively).

## 4 Discussion

### 4.1 Differential expression of gonadotropin receptors

Gonadotropin receptors mediate the biological effects exerted by gonadotropin hormones. In this study, we analyzed for the first time the expression pattern of the *fshr* and the duplicate *lhgr* during the induction of follicular maturation and ovulation of European eel using SPE treatment. The expression of the two genes encoding distinct LHCGR (*lhgr1* and *lhgr2*) was also detected in the ovary of immature eels (Maugars and Dufour, 2015). In the present case, both *lhgr1* and *lhgr2* were well expressed in the ovary during induced maturation. Since the report of the coexistence of duplicated *lhgr* in teleosts (Maugars and Dufour, 2015), their expressions have not yet been compared in any other teleost species. In fishes, there are few studies about the expression of gonadotropin receptors during follicular maturation and ovulation and none concerning gonadotropins and estradiol receptors multiple paralogs. A previous study showed a high expression of ovarian *lhgr* orthologous to eel *lhgr2*, in mature female Atlantic salmon (*Salmo salar*) caught during the upstream migration (Maugars and Schmitz, 2006), which appears characteristic of the maturation stage prior to ovulation. Moreover, we found that both genes were significantly less expressed in ovulated eggs. This agrees with the location of LH binding sites found in the granulosa and thecal cells in European eel (Salmon et al., 1988). Similarly, *lhgr* orthologous to eel *lhgr1* was found to be expressed in granulosa and weakly in theca cells in chub mackerel (*Scomber japonicus*) (Nyuji et al., 2013) and Atlantic halibut (*Hippoglossus hippoglossus*) (Kobayashi et al., 2008). Therefore, a drop in mRNA levels would be expected in successfully ovulated eggs due to the loss of the follicle layers that remain inside the ovary after the oocyte is extruded. Nevertheless, the drop in expression in ovulated eggs was more substantial for *lhgr1* (150 less expressed) than for *lhgr2* (5 times less expressed), suggesting that *lhgr1* is mostly expressed in follicular cells.

361 We hypothesized that differences in responsiveness to hormonal treatments during oocyte maturation  
362 may be related to differences in hormone receptor expression, which consequently could affect  
363 embryonic development. To test this, we calculated hatching success and larval survival as measures of  
364 embryonic developmental competence (egg quality). Transcript levels of *lhcr2* during induction of  
365 oocyte maturation were similar regardless of hatching success. However, *lhcr2* mRNA in ovulated  
366 eggs was significantly higher in the high-quality group when compared to the low-quality group,  
367 suggesting that *lhcr2* could have a role in embryonic development. Gonadotropin receptor transcripts  
368 are also present in mouse oocyte and preimplantation embryo, with a potential beneficial role in oocyte  
369 maturation and early embryonic development (Patsoula et al., 2001).

370 Previous studies have shown that gonadotropic treatment induces an increase in mRNA levels of *lhβ* as  
371 well as an increase in LH hormone in the pituitary in both European (Dufour et al., 1989; Schmitz et  
372 al., 2005) and Japanese eel (Nagae et al., 1996; Saito et al., 2003; Jeng et al., 2007). This increase in  
373 pituitary content of LH is likely necessary for the LH surge (in this case mimicked by the LH present in  
374 the SPE) triggering ovulation. However, it is still uncertain if this observation is due to the effect of  
375 gonadotropins alone, as both SPE and CPE treatments contain other components (neurotransmitters,  
376 steroids) that can affect receptor expression. Our results showed that ovarian levels of *lhcr1* at the  
377 time of SPE primer administration were both positively correlated with hatching success, and were  
378 significantly higher in the high-quality group than in the low-quality group at SPE. This suggests that  
379 females with higher hatching success had higher sensitivity to LH at the time SPE was administrated.  
380 In contrast, the significantly lower levels of *lhcr1* expression in females with low-quality eggs could  
381 have limited their response to the LH in the SPE primer. This is particularly important, in a first stage  
382 of oocyte maturation, LH regulates the follicle's ability to produce maturation-inducing steroid (MIS)

383 and the oocyte's response to MIS (i.e. oocyte maturational competence); on a second stage the follicle  
384 cells produce MIS (Patiño et al., 2001). Thus, failure to respond to LH at the first stage could  
385 subsequently compromise the oocyte's ability to respond to the MIS (i.e. the DHP injection).  
386 Additionally, LH signalling can stimulate other pathways than those leading to DHP synthesis. This has  
387 been observed in zebrafish, where the expression of *ptgs2a* (a cox-2 gene) induced by LH was  
388 necessary for ovulation to occur (Tang et al., 2017). Since oocyte maturation and ovulation may be  
389 independently/differently regulated by the same ligand, the two processes may come out of synchrony  
390 with each other, and this may influence the developmental capacity of the egg/embryo. In this case, the  
391 mismatch between *lhcr1* expression and the administration of the SPE primer may have affected the  
392 follicle's ability to respond to DHP in females in the low-quality group. Hence, a high expression of  
393 *lhcr1* before SPE priming seems to be a good biomarker of high maturational competence.

394 Overall, *fshr* was well expressed in the mature ovary at all sampling times during the induction of  
395 oocyte maturation. In Japanese eel, 17 weeks of treatment with pituitary homogenates also increased  
396 ovarian levels of *fshr* (Jeng et al., 2007). However, the physiological relevance of high *fshr* expression  
397 during ovarian maturation in teleosts is still unclear. In the case of the eel, gonadotropins contained in  
398 exogenous pituitary homogenates (SPE in this case) could up-regulate the expression of both  
399 gonadotropin receptors. Alternatively, in the case of multiple spawning, an ovulatory surge of FSH  
400 could play an important role in the follicle recruitment for the next reproductive cycle (Prat et al., 1996;  
401 Tyler et al., 1997; Sambroni et al., 2007). In this study, oocyte development presented a group-  
402 synchronous pattern (Wallace and Selman, 1981), with 2-3 cohorts of oocytes of different size and  
403 stage of development present in the ovarian biopsies taken during the induction of oocyte maturation  
404 and ovulation. Thus, a high expression of *fshr* in the ovary during maturation could be related to the

405 regulatory role of FSH on the less developed oocyte batches also present in the biopsies. Alternatively,  
406 it is also possible that FSHR has an important function on maturing oocytes (as occurs in mammals,  
407 where FSH up-regulates LHCGR (Zelevnik et al., 1974). After ovulation, levels of *fshr* mRNA level  
408 dropped below the level of detection, indicating that *fshr* is also mostly expressed in the follicle cells  
409 surrounding the oocyte. This is in agreement with the observations by *in situ* hybridization of *fshr*  
410 expression in granulosa and theca cells in vitellogenic follicles in salmon (Andersson et al., 2009).

411 In relation to hatching success, present results showed that transcript levels of *fshr* before the DHP  
412 injection were negatively correlated with hatching success, and females with low quality eggs had  
413 significantly higher transcript levels of *fshr*. This indicates that females in the low-quality group were  
414 more sensitive to FSH at the time DHP was administrated. However, while *lhβ* mRNA levels tend to  
415 increase, *fshβ* levels significantly decrease with gonadotropic treatment in both Japanese (Yoshiura et  
416 al., 1999) and European eel females (Schmitz et al., 2005). Therefore, increased sensitivity to FSH at  
417 the time of DHP injection does not appear to be determinant for successful oocyte maturation and  
418 ovulation treatment in European eel. In contrast, increasing mRNA levels of *fshr* were associated to a  
419 better competence of the oocyte to mature following pituitary hormone induction in rainbow trout  
420 (*Oncorhynchus mykiss*) (Bobe et al., 2003). However, differences in hormone receptor expression  
421 among species are likely influenced by differences in the reproductive strategies. For example, in  
422 rainbow trout which is considered as total spawner (Mylonas and Zohar, 2007), *fshr* mRNA levels peak  
423 at maturation and ovulation while *lhcg* increased significantly later after ovulation (Sambroni et al.,  
424 2007). In contrast, in zebrafish, which is a multiple batch spawner, *fshr* transcripts peaked at mid-  
425 vitellogenesis and dropped at the end of vitellogenesis (Kwok et al., 2005). Overall, a combination of  
426 low expression of ovarian *lhcg1* before SPE priming and high expression of *fshr*, when DHP is

427 administrated, could be indicators of an ineffective response to oocyte maturation treatment resulting in  
428 low egg quality.

#### 429 4.2 Differential expression of estrogen receptors

430 In this study, we quantified gene expression of three nuclear (*esr1*, *esr2a*, *esr2b*) and two membrane  
431 (*gpera* and *gperb*) estrogen receptors, during hormonal induction of oocyte maturation in European eel.  
432 We found that transcripts of *esr1* were quite similar in the eel ovary throughout induced maturation,  
433 with no significant differences between the two hatching groups. This suggests that *esr1* mRNA is not  
434 a limiting factor for the development of maturational competence. Nevertheless, up-regulation of *esr1*  
435 transcripts in the ovary of matured eels after ovulation (Lafont et al, 2015) suggests that this receptor  
436 might play an important role during oocyte maturation. Regulation of *esr1* transcript levels may be  
437 associated with an increase in circulatory levels of estradiol-17 $\beta$  (E<sub>2</sub>) during oocyte maturation stages  
438 (da Silva et al., 2016). In particular, E<sub>2</sub> plasma concentration increases two to five times after SPE  
439 priming in European eel (H. Tveiten, unpubl. results). Up-regulation of *esr1* was associated with an  
440 increase of circulatory estrogens before ovulation also *in vitro* in eel hepatocytes (Lafont et al, 2015).  
441 In contrast, *esr1* expression in the testis of male eels was markedly expressed in early stages of  
442 spermatogenesis but significantly down-regulated in late stages of spermatogenesis (Morini et al.,  
443 2017). This indicates a differential expression pattern of *esr1* between oogenesis and spermatogenesis  
444 in eels. Differences in hepatic *esr1* expression between sexes have been observed in goldfish and are  
445 likely related to differences in basal levels of E<sub>2</sub> between males and females (Nelson and Habibi, 2010).  
446 In unfertilized eggs, *esr1* transcript levels were below the level of detection, suggesting that this  
447 estrogen receptor is mostly expressed in the follicular cells.

448 Before SPE priming, *esr2a* transcripts were significantly more expressed in females with higher  
449 hatching success which may indicate that this receptor has an important role during oocyte maturation.  
450 In previous studies, the expression of *esr2a* in mature female eels after spawning was similar to  
451 controls (Lafont et al., 2015) and in male eels, it was down-regulated during final stages of  
452 spermatogenesis (Morini et al., 2017). Transcripts of *esr2a* were below the level of detection of the  
453 qRT-PCR in ovulated eggs, suggesting that they are mainly expressed in follicular cells, as *esr1*.  
454 Transcripts of *esr2b* were below the level of detection of the qRT-PCR in all samples, i.e. undetectable  
455 during follicular maturation and ovulation. In male eels, *esr2b* transcripts in the testis were significantly  
456 down-regulated throughout spermatogenesis (Morini et al., 2017). Thus, it is likely that *esr2b* does not  
457 play a key role during the final stages of gametogenesis in European eel.

458 It is well known that in female fish GPER mediates the E<sub>2</sub>-induced meiotic arrest of oocytes, e.g.  
459 zebrafish, Atlantic croaker and common carp (Majumder et al., 2015; Pang and Thomas, 2009; Pang et  
460 al., 2008; Peyton and Thomas, 2011). In these previous studies, only one GPER gene was  
461 characterized, known now to be the orthologous to teleost *gpera*. We found that *gpera* transcripts were  
462 expressed at similar levels in the ovary throughout the oocyte maturation (all stages), without  
463 significant differences between the high-quality and low-quality group. Levels of *gpera* transcript  
464 showed only a moderate decrease in ovulated eggs (not significant), indicating that *gpera* transcripts  
465 are largely present in the eel oocyte itself, in contrast to all the other estradiol receptors. In contrast,  
466 expression of *gperb* before DHP injection was significantly lower in females exhibiting higher egg  
467 viability. Furthermore, *gperb* was the only estrogen receptor that was less expressed in females  
468 exhibiting high quality eggs. This may suggest that *gperb* plays an important role in mediating the  
469 inhibitory effect of estrogen during oocyte maturation also in European eel, where estrogen-dependent

oocyte growth is arrested at the first meiotic prophase by high levels of intracellular cyclic AMP (cAMP) (Conti et al., 2002). In the pre-ovulatory phase, a LH surge causes a shift in the steroidogenic pathway to the production of the MIS. When the MIS production (DHP injection in this case) is sufficient to overcome the estrogen inhibitory effect, the binding of MIS to a G protein-coupled progesterin membrane receptor triggers meiosis resumption and GVBD by causing a decrease in cAMP concentrations (Jalabert and Finet 1986; Finet et al., 1988). Moreover, in Japanese eel, increased E<sub>2</sub> in the late stages of oocyte development inhibits the production of DHP (Ijiri et al., 1995). Thus, a lower expression of *gperb* in the HEQ group suggests a lower sensitivity to estrogen and subsequently a lower estrogen inhibition, which may have enhanced the effect of DHP. In male eels, both *gpera* and *gperb* transcripts significantly increased during spermatogenesis suggesting that GPERs may be involved in final sperm maturation (Morini et al., 2017). Overall, our results raise the possibility that an E<sub>2</sub> pathway, possibly regulated by *esr2a* and *gperb*, is involved in the control of oocyte maturation also in European eel. However, we did not find any significant correlation between the expression of these two genes and other egg quality indicators such as fertilization and hatching success. Thus, evidence is weak and further investigation is necessary to clarify their role during oocyte maturation and subsequent egg quality.

#### 4.3 Cytological indicators of oocyte maturation status

An early index of maturational acquisition is the onset of germinal vesicle migration, which is accompanied by the rise in LH at the end of follicle growth (Lubzens et al., 2010). In this study, a peripheral germinal vesicle in the most advanced oocytes was used as an indicator that females had progressed into initial stages of oocyte maturation before SPE priming. The appearance of oil droplets was used as a marker of the maturation progress to initiate SPE priming or DHP injection (Palstra et al.



2005; Unuma et al., 2011). Here, the coalescence of the oil droplets reflects the hormonal processes during final maturation resulting in decreasing numbers and increasing size of the lipid droplets. Interestingly in this study, the oocytes of females in the high-quality group had on average smaller lipid droplets with less size variation compared to those with low-quality group. Especially at DHP and ovulation the difference between the groups was significant. The observed variation in lipid droplet size, in conjunction with the hormone receptor expression pattern in the same females, suggests that treatment administrated at an earlier stage enhanced maturational competence and the subsequent developmental capacity of the egg/embryo. In general, limited information is available on the mechanisms of oocyte lipid coalescence as well as on the role of this physiologically important process in the subsequent survival and development of the eggs and embryos. Our findings, substantiating insight in the underlying hormonal processes, suggest lipid droplet diameter to be an accurate, quantitative indicator of maturation status in European eel.

In conclusion, ovarian *fshr*, *lhcr1*, *esr2a* and *gperb* were differentially expressed across sampling points between females presenting high and low egg quality. A mismatch between the timing of hormone injections and the expression of some of these genes may therefore influence the follicle's ability to respond to treatment. Furthermore, changes in hormone receptor expression were associated with changes in oocyte maturation status. Here, the average size of lipid droplets in advanced oocytes was smaller in the high- compared to low-quality group throughout the maturation process, indicating that the timing of hormonal treatments can be optimized, thereby optimizing procedures in assisted reproduction of the endangered European eel.

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737

738 **Legends**

739 **Figure 1: Oocytes at each sampling point.**

740 Photomicrographs showing examples of oocytes at the most advanced stage of development from the  
741 biopsy samples taken a) before SPE priming, b) before DHP injection and c) ovulated eggs cleared in  
742 Serra's liquid. GV: germinal vesicle and LD: lipid droplet.

743 **Figure 2: Expression of gonadotropin receptors.**

744 Boxplots representing expression of gonadotropin receptors (*fshr*, *lhcg1* and *lhcg2*) in ovarian  
745 biopsies taken at the time of administration of the SPE primer (SPE) and DHP injection (DHP) as well  
746 as ovulated eggs (EGG) in both high- (n=5) and low-quality (n=5) groups. The bold line inside each  
747 box represents the median, lower and upper sides of each box represent the lower and upper quartile  
748 (25% and 75%) and whiskers extend to the most extreme data point, no more than 1.5 times the  
749 interquartile distance. Data points outside the boxplot are classed as "outliers". Significant differences  
750 between groups are identified with "\*" when  $p < 0.05$ ; "\*\*\*" when  $p < 0.01$  and "\*\*\*\*" when  $p < 0.001$ .

751 **Figure 3: Expression of estrogen receptors.**

752 Boxplots representing expression of nuclear (*esr1*, *esr2a*) and membrane estrogen receptors (*gpera* and  
753 *gperb*) in ovarian biopsies taken at the time of administration of the SPE primer (SPE) and DHP  
754 injection (DHP) as well as in ovulated eggs (EGG) in both high- (n=5) and low-quality (n=5) groups.  
755 The bold line inside each box represents the median, lower and upper sides of each box represent the  
756 lower and upper quartile (25% and 75%) and whiskers extend to the most extreme data point, no more  
757 than 1.5 times the interquartile distance. Significant differences between groups are identified with "\*"   
758 when  $p < 0.05$  and "\*\*\*" when  $p < 0.01$ .

759 **Figure 4:**

760 Oocytes in three different maturational stages from an ovarian biopsy taken before the SPE priming  
761 injection. 1: small and opaque oocytes; 2: large oocytes with darker cytoplasm; 3: large oocytes with  
762 transparent cytoplasm and migratory nucleus.

763

764 **Figure 5: Lipid droplet diameter.**

765 Boxplot representing the lipid droplet diameter at the time of administration of the SPE priming (SPE)  
766 and DHP injection (DHP), as well as in ovulated eggs (EGG) in both high- (n=5) and low-quality (n=5)  
767 groups. The bold line inside each box represents the median, the lower and upper sides of each box  
768 represent the lower and upper quartile (25% and 75%) and whiskers extend to the most extreme data  
769 point which is no more than 1.5 times the interquartile distance. Data points outside the boxplot are  
770 classed as “outliers”. Significant differences between egg groups are identified with an “\*” when  $p <$   
771 0.05.

Figure 1

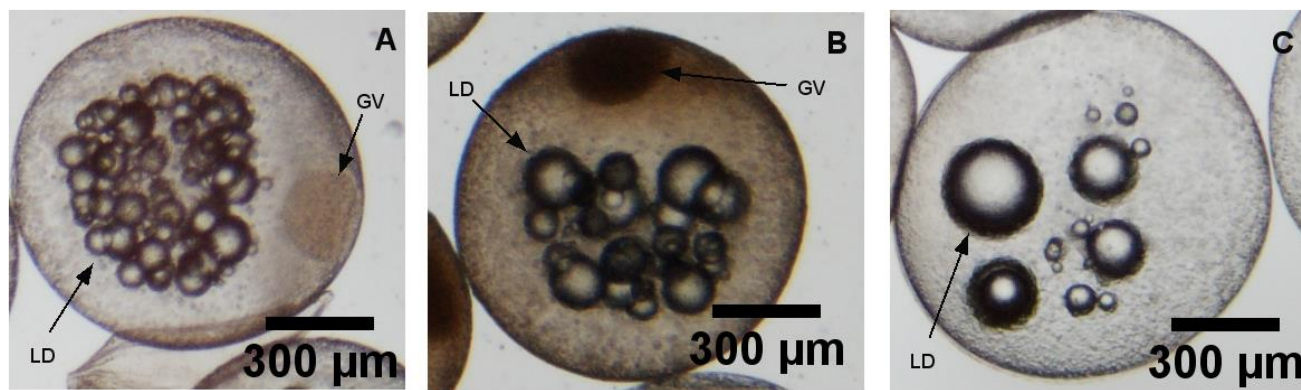


Figure 2

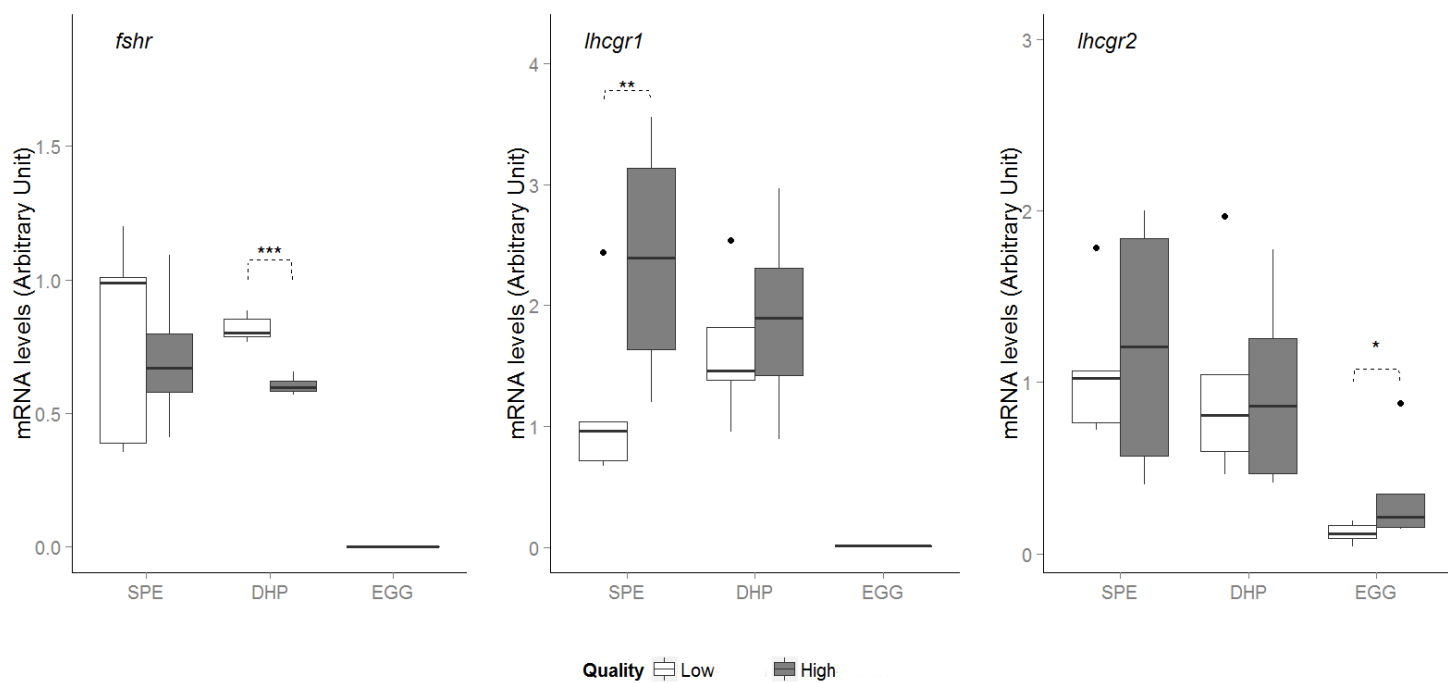


Figure 3

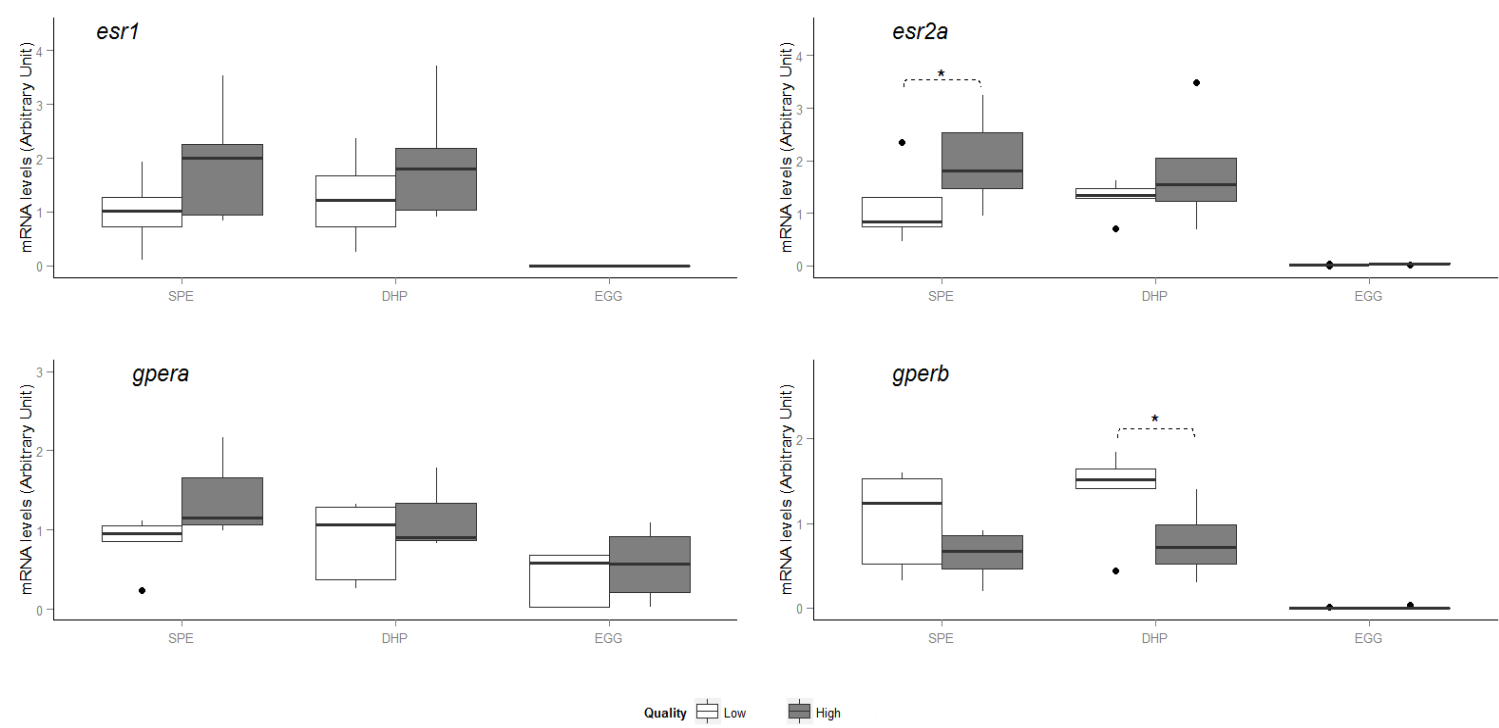


Figure 4

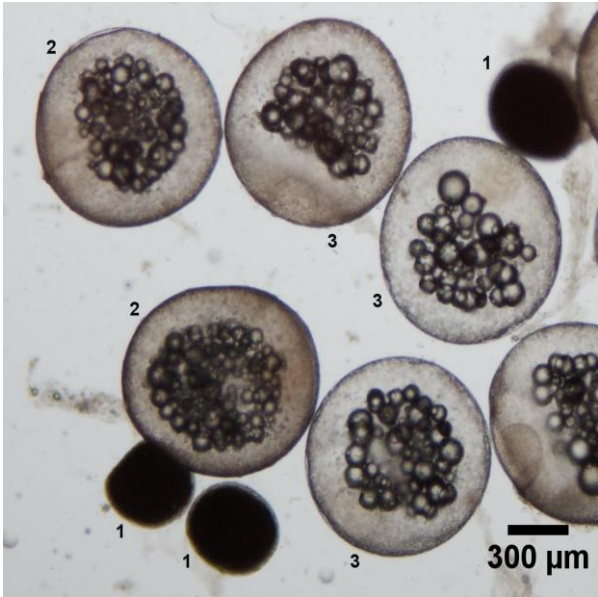
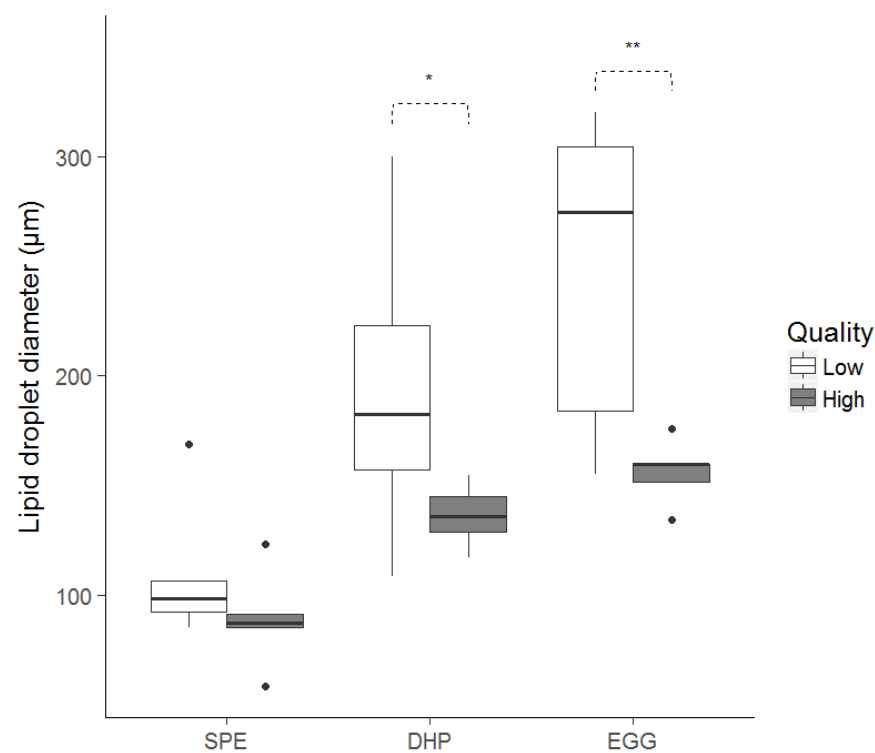


Figure 5





**Table 1** Quantitative PCR primer sequences for gonadotropin receptors (*fshr*, *lhcr1* and *lhcr2*), nuclear estrogen receptors (*esr1*, *esr2a*, *esr2b*), membrane estrogen receptors (*gpera* and *gperb*) and reference gene (*18s*).

Name	Sequence (5' - 3')	Orientation	Accession number	Amplicon size (bp)	Reference
<i>fshr</i>	CCTGGTCGAGATAACAATCACC	Forward	LN831181	148	Maugars and Dufour (2015)
	AATCTTGAGAAAATCAGGCAGT	Reverse			
<i>lhcr1</i>	GCGGAAACACAGGGAGAAC	Forward	LN831182	155	Maugars and Dufour (2015)
	GGTTGAGGTACTGGAAATCGAAG	Reverse			
<i>lhcr2</i>	GTTTCCTGACCTATCGGCTATT	Forward	LN831183	132	Maugars and Dufour (2015)
	GGTTGAGGTACTGGAAATCGAAG	Reverse			
<i>esr1</i>	GCCATCATACTGCTCAACTCC	Forward	CUH82767	76	Lafont et al., (2015)
	CCGTAAAGCTGTCGTTTCAGG	Reverse			
<i>esr2a</i>	TGTGTGCCTCAAAGCCATTA	Forward	CUH82768	169	Lafont et al., (2015)
	AGACTGCTGCTGAAAAGGTCA	Reverse			
<i>esr2b</i>	TGCTGGAATGCTGCTGGT	Forward	CUH82769	123	Lafont et al., (2015)
	CCACACAGTTGCCCTCATC	Reverse			
<i>gpera</i>	CAACTTCAACCACCGGGAGA	Forward	CUH82770	170	Lafont et al., (2015)
	TGACCTGGAGGAAGAGGGACA	Reverse			
<i>gperb</i>	AACCTGAACCACACGGAAA	Forward	CUH82771	170	Lafont et al., (2015)
	TGACCTGGAAGAAGAGGGACA	Reverse			
<i>18s</i>	CTCAACACGGGAAACCTCAC	Forward	FM946070	118	
	AGACAAATCGCTCCACCAAC	Reverse			

**Table 2** Information and group assignment about female eels and resulting egg batches used in the present study, including initial length, ( $L_i$ ) and body weight ( $BW_i$ ), volume of eggs stripped, floating fraction (%), fertilization success (%), hatching success (%) and larval survival (%) at 3 days post hatch (dph).

Female No.	$L_i$ (cm)	$BW_i$ (g)	Eggs (g)	Floating	Fertilization	Hatching	Survival 3 dph	Group
1.	70	693	322	99	68	65	67	high-quality
2.	67	574	324	99	99	80	93	high-quality
3.	60	433	229	99	69	55	83	high-quality
4.	78	1014	335	95	97	19	13	high-quality
5.	86	1444	571	50	80	67	78	high-quality
6.	79	1019	372	25	84	4	0	low-quality
7.	63	414	200	90	15	5	0	low-quality
8.	92	1334	586	99	20	0	0	low-quality
9.	62	426	195	50	25	0	0	low-quality
10.	60	459	133	100	96	4	0	low-quality

**Table 3** Pearson's correlation coefficient R and *p*-value for the linear regressions between gene expression at the time of SPE priming (SPE), DHP injection (DHP) and ovulated eggs (EGG), and hatching and fertilization success (n=10).

Gene	Time point	Hatching		Fertilization	
		R	<i>p</i>	R	<i>p</i>
<i>fshr</i>	SPE	0.23	0.529	-0.20	0.577
	DHP	-0.79	0.012	-0.46	0.213
	EGG	NA	NA	NA	NA
<i>lhcr1</i>	SPE	0.68	0.032	0.10	0.780
	DHP	0.36	0.314	-0.38	0.281
	EGG	-0.17	0.637	-0.17	0.636
<i>lhcr2</i>	SPE	0.00	0.999	0.42	0.223
	DHP	-0.24	0.512	0.40	0.250
	EGG	0.05	0.892	0.32	0.397
<i>esr1</i>	SPE	0.54	0.111	-0.11	0.762
	DHP	0.41	0.236	-0.33	0.351
	EGG	NA	NA	NA	NA
<i>esr2a</i>	SPE	0.60	0.067	0.05	0.894
	DHP	0.49	0.154	-0.11	0.762
	EGG	0.60	0.064	0.35	0.316
<i>gpera</i>	SPE	0.34	0.331	0.56	0.094
	DHP	0.29	0.419	0.46	0.183
	EGG	0.06	0.865	0.38	0.275
<i>gperb</i>	SPE	-0.44	0.204	-0.62	0.058
	DHP	-0.31	0.413	-0.21	0.584
	EGG	NA	NA	NA	NA